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# N-acylphosphatidylethanolamine, a Gut-Derived Circulating Factor Induced by Fat Ingestion, Inhibits Food Intake

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## SUMMARY

N-acylphosphatidylethanolamines (NAPEs) are a relatively abundant group of plasma lipids of unknown physiological significance. Here, we show that NAPEs are secreted into circulation from the small intestine in response to ingested fat and that systemic administration of the most abundant circulating NAPE, at physiologic doses, decreases food intake in rats without causing conditioned taste aversion. Furthermore, $^{14}$C-radiolabeled NAPE enters the brain and is particularly concentrated in the hypothalamus, and intracerebroventricular infusions of nanomolar amounts of NAPE reduce food intake, collectively suggesting that its effects may be mediated through direct interactions with the central nervous system. Finally, chronic NAPE infusion results in a reduction of both food intake and body weight, suggesting that NAPE and long-acting NAPE analogs may be novel therapeutic targets for the treatment of obesity.

## INTRODUCTION

Under normal conditions, appetite is regulated largely by the gut-brain-adipocyte axis, an intricate circuit that balances energy intake with energy expenditure to promote the maintenance of energy reserves (Abizaid et al., 2006; Morton et al., 2006). Hormones, nutrients and vagal afferents interact with targets in the brainstem, hypothalamus, and midbrain to modify the activity of neuronal populations that regulate food intake and energy expenditure in both short- and long-term contexts (Cone et al., 2001). Due to its fenestrated capillaries, the hypothalamic arcuate nucleus (ARC) is well-suited to detect endocrine and nutrient factors that regulate food intake (Fry and Ferguson, 2007). Orexigenic *NPY* and anorexigenic *POMC* ARC neurons respond by communicating with second-order regulatory neurons in the paraventricular nucleus (PVN) and lateral hypothalamus (LH) to mediate the appropriate feeding behavior (Schwartz et al., 2000).

In addition to integrating the effects of leptin, ghrelin, insulin, and gastrointestinal peptides including CCK, $PYY_{3-36}$, and GLP-1, cells of the ARC regulate food intake by directly sensing nutrients (Obici et al., 2002; Abizaid et al., 2006; Coll et al., 2007). Peripheral or central infusions of glucose suppress food intake, and disruption of glucose transport or its intracellular metabolism elicit hyperphagia in experimental animals (Miselis and Epstein, 1975; Wolfgang et al., 2007). Similarly, intracerebroventricular (ICV) administration of L-leucine (Cota et al., 2006) reduces food intake in rodents, suggesting that the central nervous system (CNS) can sense short-term glucose and amino acid availability independently of hormones secreted by the gastrointestinal tract.

Neuronal sensing of fatty acids (FAs) has also been implicated in the regulation of feeding behavior by studies demonstrating that food intake can be reduced through inhibition of fatty acid synthase (Loftus et al., 2000) or intrathecal oleic acid infusion (Obici et al., 2002). However, in contrast to plasma concentrations of glucose and amino acids, which increase after meals, concentrations of plasma FAs typically decrease with feeding and rise with fasting, contrary to what is expected of a negative regulator of appetite (Dole, 1956). Furthermore, intravenous infusions of lipid do not affect food intake, and thus it remains unclear how either circulating triglyceride (TG) or FAs signal lipid surfeit to the CNS under physiological conditions (Little et al., 2007).

Given that other types of macronutrients communicate directly with cells in the hypothalamus, we hypothesized that food intake could be modified by circulating nutrient signals reflective of meal fat content. To examine this hypothesis, we undertook a screen of lipid derivatives that increased in plasma after high-fat feeding using liquid chromatography tandem mass spectrometry (LC/MS/MS). Among these increased metabolites was a class of phospholipids, the N-acylphosphatidylethanolamines (NAPEs), of previously unknown physiologic function in

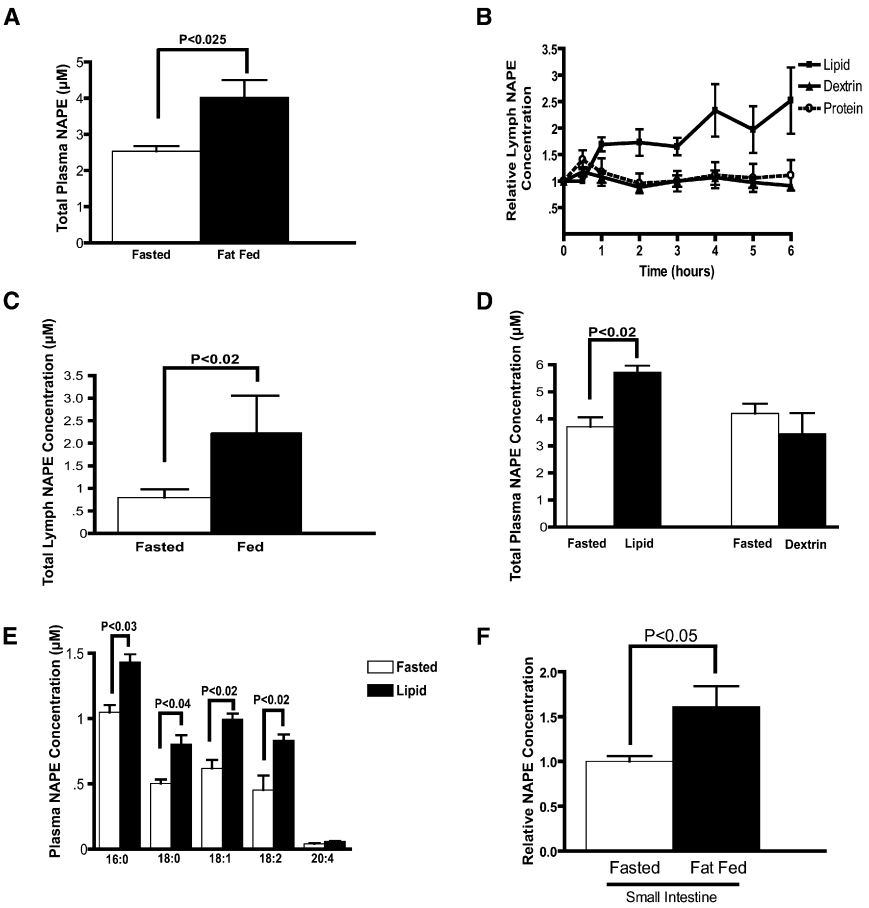


**Figure 1. Regulation of NAPE Levels in Rat Plasma, Lymph and Small Intestine by Fat Feeding and Intraduodenal Lipid Infusion**

(A) High-fat chow feeding increases total plasma NAPE ($p < 0.025$) at 4 hr relative to fasting conditions ($n = 8$ per group).

(B) Time course of intraduodenal lipid (solid squares), dextrin (filled triangles), or protein (open circles, dashed line) infusions' effects on relative lymph NAPE concentration ($p < 0.0025$) ($n = 4–8$ per group).

(C) Intraduodenal infusion of lipid significantly increases absolute NAPE concentration in lymph 4 hr after the start of the experiment ($p < 0.02$) ($n = 7–8$ per group).

(D) Intraduodenal infusion of lipid, but not dextrin, increases plasma NAPE concentration ($p < 0.02$) 4 hr after the start of the experiment ($n = 4$ per group).

(E) N-acyl species profile of plasma NAPE at 4 hr after the start of the lipid infusion (solid bars) compared with fasting levels in the same animals (open bars) ($n = 4$ per group).

(F) High-fat feeding significantly increases NAPE content in the small intestine relative to fasting ($p < 0.05$) ($n = 4$ per group).

All data are expressed as mean ± SEM.

plasma, whose hydrolysis products, the N-acylethanolamines, have been implicated in the peripheral control of food intake (Rodriguez de Fonseca et al., 2001; Fu et al., 2003). We found that intraperitoneal and intravenous injection of the most abundant plasma NAPE, at physiologic doses, reduced food intake.

## RESULTS

### Fat Feeding Increases NAPE Levels in Lymph and Plasma

When fasted rats were fed a high-fat diet, we observed a 60% increase in total plasma NAPE concentrations (Figure 1A). In order to confirm that dietary lipids were responsible for this increase, we subjected rats to intraduodenal infusions of lipid (Liposyn II), a glucose polymer (dextrin), or protein (casein) and monitored NAPE changes in lymph. Within 60 min of initiating the lipid infusion, we found that lymph NAPE increased by more than 50% and remained elevated for the duration of the 6 hr experiment (Figures 1B and 1C). In contrast, there were no significant increases in lymph NAPE concentrations after dextrin or protein administration in an identical infusion protocol (Figure 1B).

We next examined the effects of lipid or dextrin infusion on NAPE concentrations in plasma. Consistent with the results from the lymph studies, we found that lipid, but not dextrin, infusion increased plasma NAPE levels by approximately 40% within 1 hr of the start of the lipid infusion and maintained them at elevated levels for the duration of the experiment (Figure 1D). In order to determine which acyl species of NAPE increased after lipid infusion, we analyzed plasma samples from fasted and lipid-infused rats with LC/MS/MS. We found that 16:0, 18:0, 18:1, and 18:2 NAPEs all increased by more than 50% in plasma, whereas levels of the endocannabinoid precursor 20:4 NAPE remained unchanged (Figure 1E). Because C16:0 is the most abundant circulating NAPE and the most increased by fat infusion, it was chosen for subsequent studies.

### Fat Feeding Increases NAPE Content in the Small Intestine

Since the fatty acid components of NAPE that appeared in the plasma and lymph after lipid infusion were similar to the fatty acid composition of the infused lipid, we reasoned that the plasma and lymphatic NAPE was derived from the infused lipid and synthesized in the gut. In order to test this hypothesis and determine where in the gastrointestinal tract NAPE might be synthesized, we examined NAPE content in the stomach, small intestine, and large intestine in rats that had either been fasted or refed with a high-fat diet. We found that NAPE content increased in the small intestine after fat feeding (Figure 1F) but did not change in the stomach or colon (Figure S1 available online). These data are consistent with previous studies demonstrating NAPE synthesis in small intestinal gut segments with feeding (Petersen et al., 2006; Fu et al., 2007).

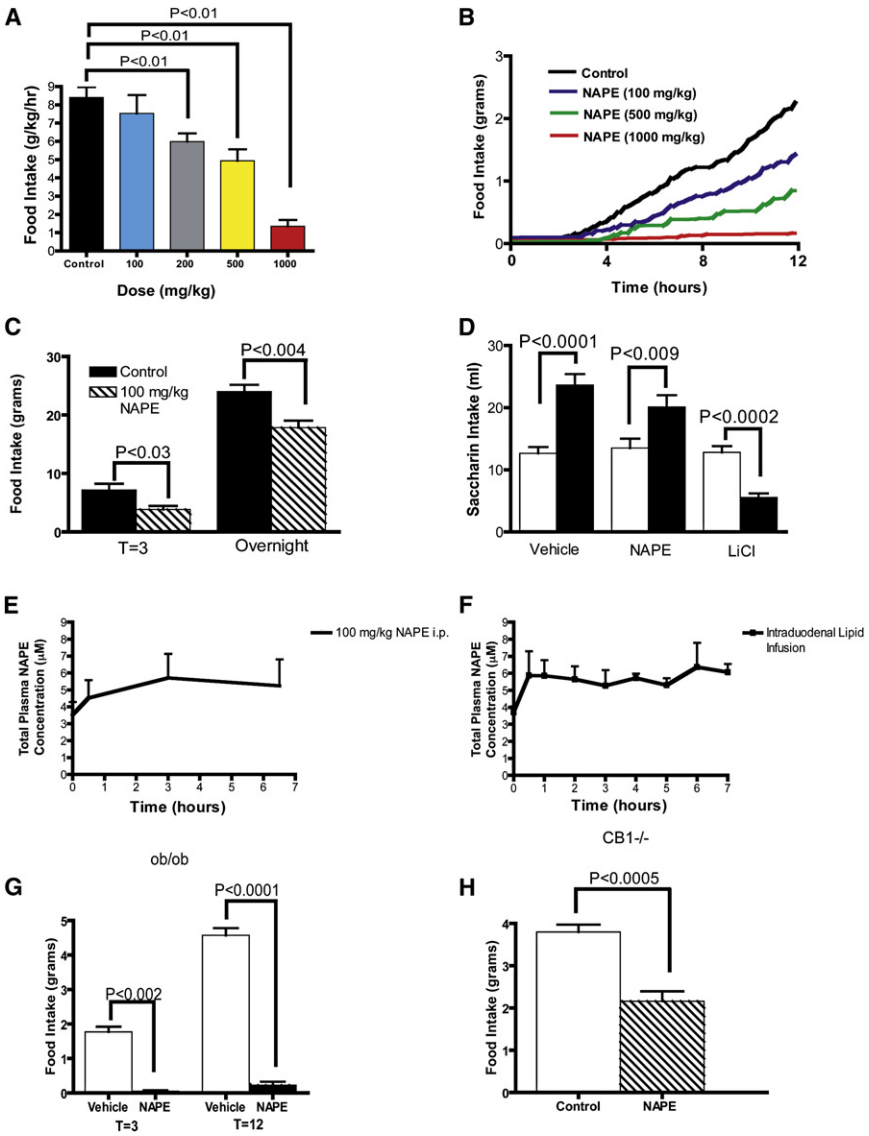


**Figure 2. C16:0 NAPE Treatment Suppresses Food Intake Dose Dependently without Causing Conditioned Taste Aversion**

(A) Systemic (i.p) NAPE administration significantly reduces the rate of food intake in a dose-dependent manner at 200 mg/kg, 500 mg/kg, and 1000 mg/kg ($p < 0.01$) in free-feeding mice ($n = 4–14$ per group).

(B) Systemic (i.p) NAPE administration reduces overnight cumulative food intake dose-dependently in free-feeding mice ($n = 4–11$ per group).

(C) Treatment with 100 mg/kg C16:0 NAPE (i.p.) significantly reduces overnight food intake in ad libitum-fed rats at t = 3 hr ($p < 0.03$) and overnight t = 13 ($p < 0.004$) ($n = 6$ per group).

(D) Treatment with 100 mg/kg C16:0 NAPE (i.p.), a dose sufficient to reduce overnight food intake, does not produce conditioned taste aversion in rats, in contrast to 100 mg/kg LiCl. Open bars indicate the amount of saccharin solution consumed when presented to 24 hr water-deprived, naive rats, and filled bars show the amount of saccharin solution consumed in 24 hr water deprived rats trained to associate the sweet taste with saline, C16:0 NAPE, or LiCl ($n = 6$ per group).

(E) Total plasma NAPE time course in fasted rats injected with 100 mg/kg C16:0 NAPE at t = 0 ($n = 5–9$ per time point).

(F) Total plasma NAPE time course in fasted rats given an intraduodenal infusion of lipid (Liposyn II) at t = 0 ($n = 4$ per group).

(G) C16:0 NAPE (500 mg/kg) dramatically suppresses overnight food intake in ad libitum-fed *ob/ob* mice at t = 3 ($p < 0.002$) and t = 12 ($p < 0.0001$) ($n = 4$ per group).

(H) C16:0 NAPE (500 mg/kg) suppresses overnight food intake in fasted $CB1^{-/-}$ mice ($p < 0.0005$) ($n = 5$ per group).

All data are expressed as mean ± SEM.

## The Most Abundant Plasma NAPE Reduces Food Intake in Rats and Mice without Causing Taste Aversion

We next examined whether exogenous administration of NAPE could alter food intake. We injected rats and mice intraperitoneally with varying doses of C16:0 NAPE and monitored food intake and activity in metabolic cages. We found that NAPE treatment reduced food intake in a dose-dependent manner, whereas an isovolumetric dose of lipid had no such effect (Figure 2A). Furthermore, food intake was almost completely suppressed at the highest dose of NAPE, and its anorexigenic effects at this dose persist for 12 hr (Figure 2B).

In rats, C16:0 NAPE (100 mg/kg i.p.) given immediately before the onset of the dark cycle, the interval during which rats consume the bulk of their daily calories, significantly reduced food intake (Figure 2C). Importantly, this dose of NAPE, as well as a higher dose of NAPE (250 mg/kg i.p.) and intracerebroventricular infusion of NAPE (80 nmol) did not cause conditioned taste aversion (CTA), unlike the noxious agent lithium chloride (Figure 2D and Figure S2).

## Fat Feeding and 100 mg/kg NAPE Treatment Produce Similar Increases in Plasma NAPE Levels

Because many compounds of endogenous origin affect feeding behavior when administered in sufficient quantities, we sought to test the physiological significance of NAPE as a regulator of food intake. Measurement and comparison of plasma NAPE levels after 100 mg/kg i.p. injection of C16:0 NAPE or intraduodenal bolus of Liposyn II at t = 0 (Figures 2E and 2F) indicate that both the absolute increase in circulating NAPE and the time course of that increase are similar between the lipid-fed and NAPE-injected groups. Changes observed in mice treated with NAPE (100 mg/kg i.p.) were similar, increasing from 3.8 ± 0.3 μM at baseline to a peak of 12 ± 2.6 μM at 3 hr after injection ($n = 5$ per group, data not shown).

### *ob/ob* Mice are Hypersensitive to Exogenous NAPE Administration

To investigate the possibility that the actions of NAPE may exert its effects through leptin signaling, we tested its effects on leptin-deficient *ob/ob* mice. Surprisingly, we found that the *ob/ob* mice were actually more sensitive than wild-type controls to the satiating effects of exogenously administered NAPE (Figure 2G).

### *CB1*$^{-/-}$ Mice Respond Normally to Exogenous NAPE Administration

Considering NAPE's structural similarity to identified endogenous CB1 agonists, we also examined whether the effects of NAPE require the CB1 receptor by studying NAPE treatment in *CB1*$^{-/-}$ mice. We found that NAPE treatment was still effective in suppressing overnight food intake in *CB1*$^{-/-}$ mice, suggesting that NAPE reduces food intake independently of the CB1 receptor (Figure 2H).

### Central NAPE Administration Reduces Food Intake

Because the vast majority of appetite-regulatory factors have targets in the CNS, we tested the hypothesis that NAPE effects on food intake are centrally mediated. To do this, we injected C16:0 NAPE (80 nmol) into the lateral ventricle of chronically cannulated (ICV) mice and recorded overnight food intake relative to ICV lipid-treated (Liposyn II) control mice in metabolic cages. We found that ICV injection of C16:0 NAPE reduced food intake by 56% at 12 hr relative to lipid-treated animals (Figure 3A). In a second control experiment, we found that central infusion of an isomolar quantity of another phospholipid, dioleoylphosphatidylethanolamine (DOPE), had no effect on food intake (Figure 3B).

### NAPE Reduces Food Intake in Vagotomized Rats

Although the above investigation indicated central activity, we sought to exclude the possibility that vagal afferents were responsible for NAPE-induced hypophagia by examining the effects of systemic NAPE injection in rats that were either subjected to a bilateral subdiaphragmatic vagotomy or a sham procedure in which the vagus nerve was exposed but not severed. When given identical doses of C16:0 NAPE (100 mg/kg), the two groups were found to have similar NAPE-induced reductions in food intake (Figure 3C, sham group, data not shown). The surgical procedure itself caused no differences in long term food intake or body weight (Figure S3).

### $^{14}$C-NAPE Crosses the Blood-Brain Barrier and Is Concentrated in the Hypothalamus

To examine whether plasma C16:0 NAPE is able to enter the central nervous system, we infused catheterized rats with a bolus of $^{14}$C-NAPE and measured $^{14}$C counts in the brain and hypothalamus 4 hr after treatment. After $^{14}$C-NAPE infusions, we found significant increases in $^{14}$C counts in the brain and more importantly a more than 5-fold increase in $^{14}$C counts in the hypothalamus. This suggests that at minimum, the N-acylethanolamine (NAE) head group of NAPE is capable of entering the CNS from peripheral circulation and that it preferentially accumulates in the hypothalamus (Figure 3D).

### Central Administration of Palmitoylethanolamide Does Not Affect Food Intake

Because NAPEs are precursors of NAEs, which have been shown to influence food intake, we also infused the hydrolysis product of C16:0 NAPE, palmitoylethanolamide (PEA) directly into the lateral ventricle of cannulated mice. In contrast to NAPE, PEA did not suppress food intake overnight when administered in this manner (Figure 3E). Moreover, treatment of mice (i.p.) with an inhibitor of NAE hydrolysis, URB579, at a dose sufficient to quadruple NAE concentrations in the CNS, did not affect overnight food intake in fasted mice (Figure 3F). Taken together, these data are consistent with previous studies demonstrating no effect of intrathecal administration of the NAE oleoylethanolamide (OEA) on food intake (Rodriguez de Fonseca et al., 2001).

### Central and Peripheral NAPE Administration Reduces Voluntary Locomotor Activity

In addition to the dose-dependent effect of NAPE to reduce food intake, we also found that NAPE administration caused a dose-dependent reduction in activity (Figure 4A) reminiscent of the rodent behavioral satiety sequence characterized by postprandial grooming and rest (Halford et al., 1998). To exclude the possibility that this reduction in activity may have physically prevented animals from gaining access to food, we examined the effect of NAPE treatment on mouse performance on an accelerating rotarod, a well-validated measure of motor performance (Carter et al., 1999). Pretrained mice treated with the highest tested dose of C16:0 NAPE performed as well as mice that received vehicle, suggesting that the reduction in movement elicited by NAPE treatment is voluntary (Figure 4B). We next examined whether ICV administration of C16:0 NAPE in mice had similar effects on locomotor activity as did peripheral treatments, and we found that very small quantities of ICV administered NAPE were sufficient to reproduce the behavioral effects obtained from much larger doses of NAPE given by intraperitoneal injection (Figure 4C).

### NAPE Treatment Reduces Fasting-Induced Increases in *NPY* Neuron cFOS Expression

Neuropeptide Y (NPY) stimulates feeding and increases behavioral arousal (Stanley and Leibowitz, 1985; Sahu et al., 1988; Kalra et al., 1999). Because NAPE treatment decreased food intake and activity, we hypothesized that its effects might be mediated by *NPY* neurons in the hypothalamic ARC. We tested this hypothesis by quantifying immediate early gene *cFOS* expression in fasted transgenic mice expressing green fluorescent protein (GFP) in *NPY* neurons with and without NAPE treatment. Fasting increases NPY activity in the ARC, compared to the fed state, as reflected by a 4-fold increase in cFOS expression in these cells (Figures 5A, 5B, and 5G). With intraperitoneal NAPE treatment, however, cFOS activity in *NPY* neurons was suppressed by 64%, to a level comparable to that observed in the free-feeding ARC (Figures 5C, 5G, and 5H). In addition, we found that the canonical fasting-induced cFOS expression throughout the ARC was decreased by NAPE treatment, suggesting that increases in systemic NAPE may also suppress

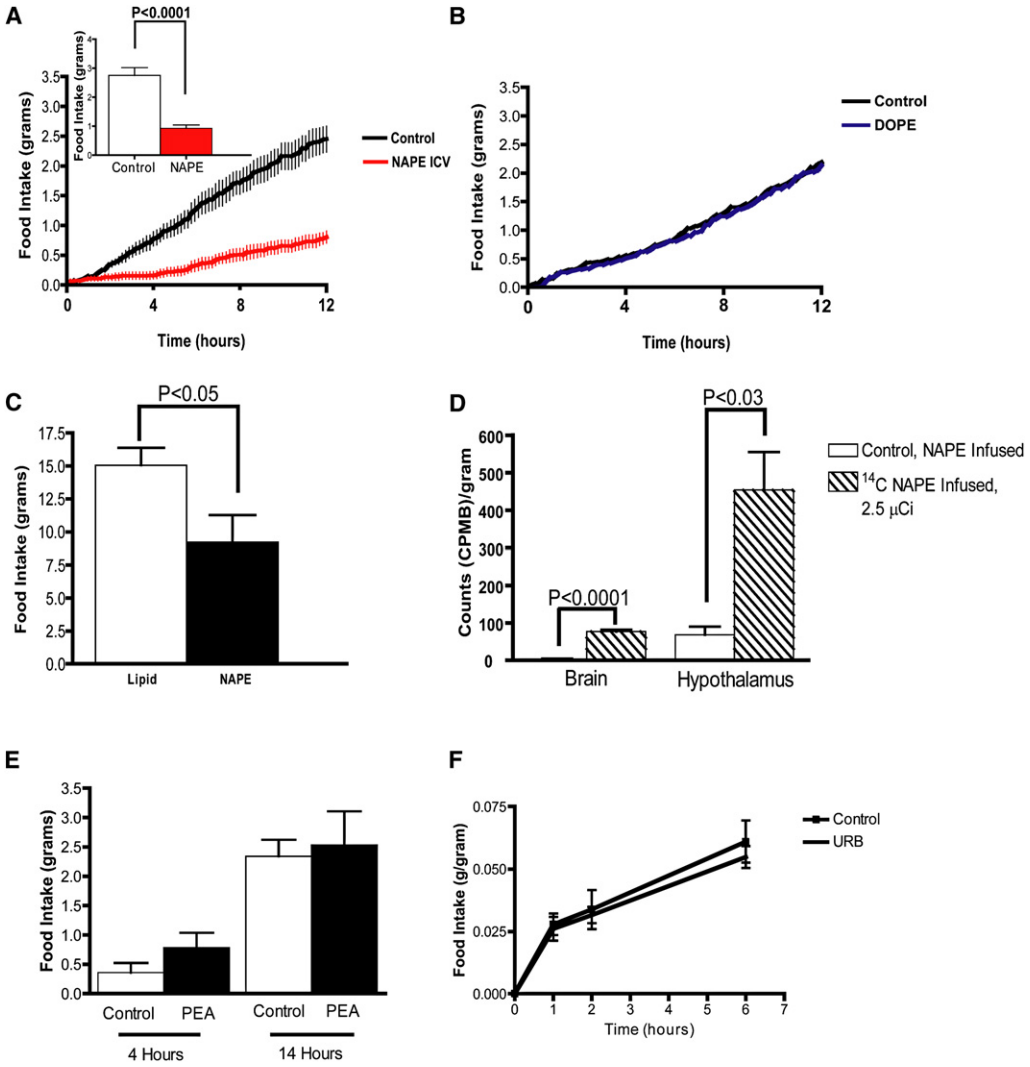


**Figure 3. C16:0 NAPE Treatment Suppresses Food Intake by a Central Mechanism**

(A) Central (i.c.v.) administration of C16:0 NAPE (80 nmol) significantly ($p < 0.0001$) reduces overnight food intake in mice relative to lipid control (Liposyn II) ($n = 6–7$ per group).

(B) i.c.v. administration of 80 nmol dioleoylphosphatidylethanolamine (DOPE), a phospholipid, to mice does not reduce food intake relative to lipid control (Liposyn II) ($n = 4$ per group).

(C) C16:0 NAPE (100 mg/kg i.p.) reduces overnight food intake in vagotomized rats, indicating that its suppressive effects on food intake do not require intact vagal afferents ($p < 0.05$) ($n = 5–8$ per group).

(D) Intravenous infusion of 2.5 μCi $^{14}$C NAPE significantly increases counts in the brain ($p < 0.0001$) and hypothalamus ($p < 0.03$) of treated animals relative to controls at $t = 4$ ($n = 5–6$ per group).

(E) Central administration of PEA, the C16:0 NAPE hydrolysis product (100 nmol), does not affect overnight food intake in mice ($n = 6–7$ per group).

(F) Systemic pretreatment with URB597 (0.3 mg/kg), an inhibitor of central NAE degradation, which increases brain NAE concentrations, does not affect food intake in fasted mice ($n = 4$ per group).

All data are expressed as mean ± SEM.

fasting-elicited transcriptional activity in other neural networks in the hypothalamus (Figures 5D–5F and 5I).

## NAPE Treatment Stimulates cFOS Expression in the Paraventricular and Supraoptic Nuclei of the Hypothalamus

cFOS staining performed in brain slices obtained from other areas demonstrated rapid and dramatic activation of neurons in the PVN (Figures 5J–5L) and supraoptic nucleus (SO) (Figure S4) in response to NAPE treatment relative to fasting.

## Chronic High-Fat Feeding Eliminates Postprandial NAPE Secretion but Not Sensitivity to Exogenous NAPE

In order to examine whether alterations in NAPE production, or perturbed NAPE signaling, may play a role in diet induced obesity, we fed rats either a high-fat diet (HFD) or a regular

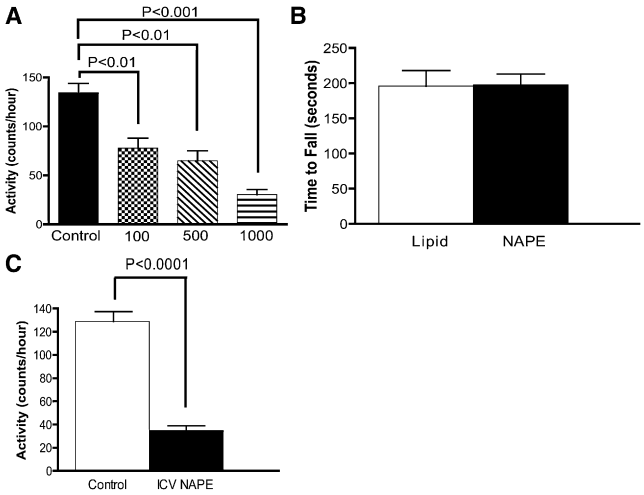


**Figure 4. Central and Peripheral NAPE Treatment Reduces Spontaneous Activity**

C16:0 NAPE administration (i.p.) reduces locomotor activity without causing motor deficits, and central administration of a small quantity of i.c.v. NAPE is sufficient to reproduce this effect.

(A) Systemic C16:0 NAPE administration produces a dose-dependent reduction in motor activity relative to lipid control at 100 mg/kg ($p < 0.01$), 500 mg/kg ($p < 0.001$), and 1000 mg/kg ($p < 0.001$) ($n = 4–12$ per group).

(B) Systemic (i.p.) NAPE administration (1000 mg/kg) does not affect time to fall from the accelerating rotarod, a test of motor coordination and ability to respond to a motor challenge in pretrained mice ($n = 5$ per group).

(C) Central administration (i.c.v.) of C16:0 NAPE, 80 nmol, or 0.25% of the maximum dose (1000 mg/kg) given peripherally causes a similar reduction in locomotor activity ($p < 0.0001$) ($n = 3$ per group).

All data are expressed as mean ± SEM.

chow (RC) diet for 1 month, implanted jugular venous catheters, allowed for postoperative recovery, and then measured NAPE secretion in plasma after a high-fat meal after an overnight fast. As we have shown, animals maintained on RC showed a marked increase in plasma NAPE concentrations after the high-fat meal.

Surprisingly, animals fed with HFD for 35 days prior to the experiment, although exhibiting high to normal NAPE concentrations at baseline, were unable to induce secretion postprandially and even showed a significant reduction in circulating NAPE during the postmeal interval (Figure 6A). Despite this derangement in NAPE synthesis and/or secretion after fat feeding, the chronically high fat-fed animals remained responsive to the anorectic effects of C16:0 NAPE in separate feeding studies (Figure 6B).

### Continuous Low-Dose NAPE Infusion Reduces Food Intake and Body Weight

Finally, we examined whether chronic C16:0 NAPE administration would have an effect on energy balance and food intake in a multiday experiment. We infused vehicle or C16:0 NAPE (0.07 mg/kg/min) intravenously in chronically catheterized free-ranging rats while recording daily food intake and body weight. After 5 days of treatment, we found that cumulative food intake in the NAPE-treated rats was reduced by approximately 30% compared to the vehicle infused rats (Figures 7A and 7C) and that the consequence of this hypophagia was significant weight loss in the NAPE group without any other behavioral changes (Figure 7B).

## DISCUSSION

Because dietary fat is the most obesity-promoting macronutrient, it has been identified as an important causal factor in the obesity epidemic (Donahoo et al., 2008). Therefore, understanding the processes by which dietary fat intake is regulated and how these mechanisms malfunction in our modern environment may lead to new therapeutic options for the treatment of obesity. In this paper, we present data suggesting that circulating NAPEs may be involved in the physiologic regulation of dietary fat intake.

In contrast to peptide mediators of satiety or satiation like CCK and GLP-1 that are released by endocrine cells of the gastrointestinal tract in response to multiple types of ingested of macronutrients (Little et al., 2007), we found that secretion of NAPEs into plasma and lymph requires the consumption of dietary lipid. Because NAPE levels are unchanged in the stomach and large intestine with nutrient intake, and because studies using labeled precursors have shown that intestinal tissues readily synthesize NAPE (Rodriguez de Fonseca et al., 2001; Petersen et al., 2006), these data suggest that the small intestine is the major source for the increase in lymph and plasma NAPEs seen under these conditions of fat feeding.

When administered systemically to rats and mice, C16:0 NAPE reduced food intake in a dose-responsive fashion. Importantly, a 100 mg/kg i.p. dose of C16:0 NAPE caused significant reduction in food intake while increasing plasma NAPE levels to a similar extent as that observed after intraduodenal lipid infusion. This suggests that the reduction in food intake at this dose is physiologic and that NAPE generation and secretion into circulation may be one means by which the gut conveys information about lipid availability in the digestive tract to the central nervous system, likely in concert with increased NAE signaling in the small intestine (Fu et al., 2007). Furthermore, our finding that NAPEs circulate in plasma at much higher concentrations than NAEs (low μM versus low nM range) may explain why many of the targets activated by NAEs, including the anorexigenic G protein-coupled receptor GPR119, require apparently supraphysiological concentrations of NAE ligand to respond (Overton et al., 2006). If NAPEs can also activate these receptors, then this apparent paradox might be resolved. It is also important to note that at 100 mg/kg or 250 mg/kg, C16:0 NAPE did not cause conditioned taste aversion, a behavioral response elicited in experimental animals by compounds that cause nausea or general malaise, supporting the possibility that NAPE is a physiologic regulator of food intake. However, because intraperitoneal, but not intravenous, NAPE treatment can produce transient, nonaversive postural changes in rats, but not mice, during the immediate postinjection period, the latter mode of administration is preferable and most physiologic. Regardless, both routes of administration had the same net effect on food intake and energy balance.

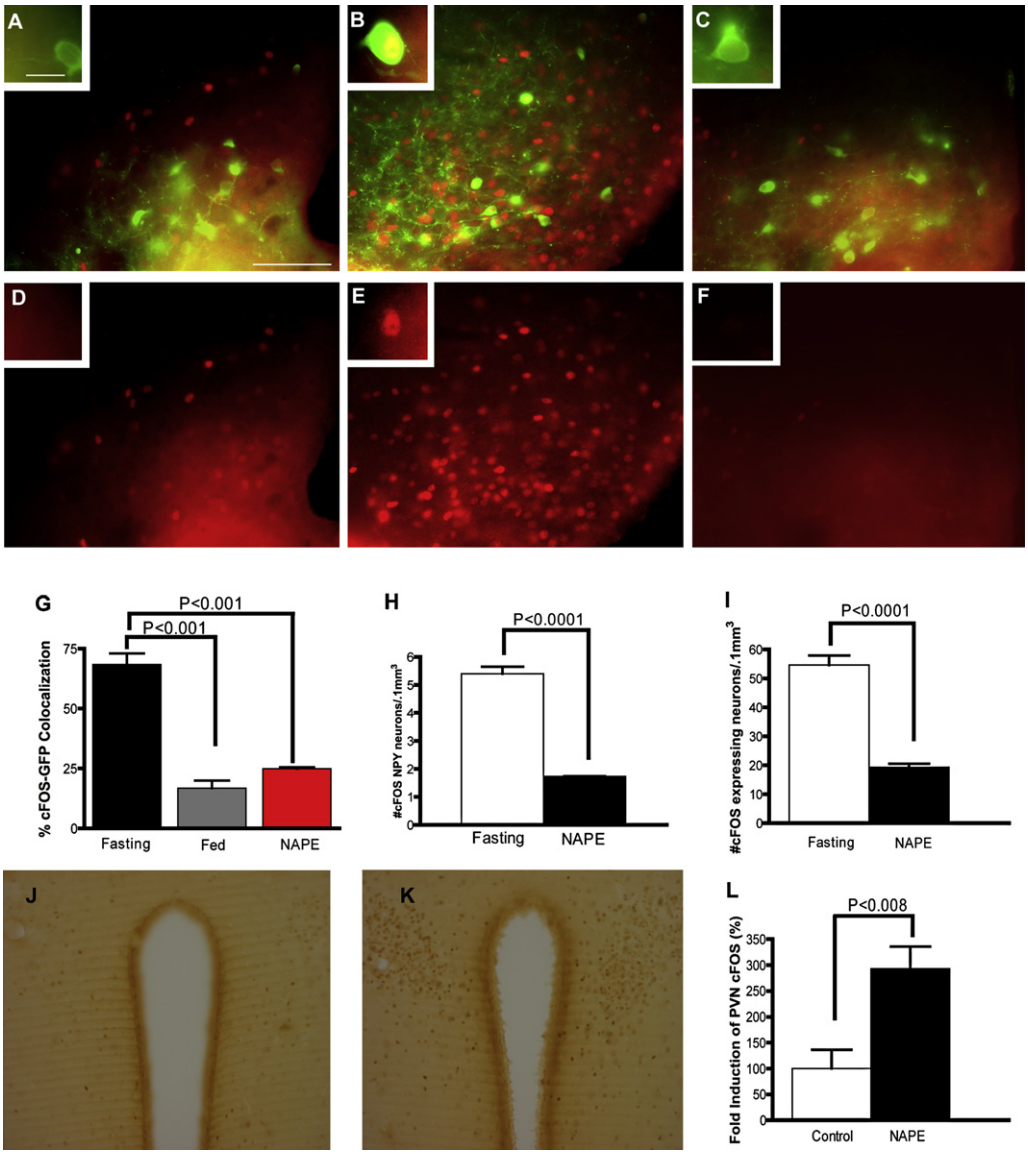


**Figure 5. Peripheral NAPE Treatment Antagonizes Fasting-Evoked cFOS Changes in the Hypothalamus**

Systemic C16:0 NAPE treatment (1000 mg/kg i.p.) reduces fasting-induced activation of *NPY* neurons in the hypothalamic ARC of transgenic mice expressing GFP in *NPY* neurons and increases cFOS expression in the hypothalamic PVN. cFOS is stained in red.

(A) cFOS-GFP colocalization in the arcuate nucleus of free-feeding mice is low, indicating few transcriptionally active *NPY* neurons (small scale bar, inset, 10 μm; large scale bar, panel, 100 μm).

(B) Overnight fasting dramatically increases colocalization, indicating activated *NPY* neurons.

(C) C16:0 NAPE treatment of fasted animals results in reduction of NPY-GFP colocalization to fed levels.

(D) Diffuse expression of cFOS is low in the ARC of free-feeding animals.

(E) Overnight fasting also stimulates cFOS expression in GFP-negative cells throughout the ARC.

(F) C16:0 NAPE treatment markedly reduces cFOS expression in the ARC of fasted animals.

(G) Quantification of percent cFOS-GFP colocalization in the arcuate nucleus of all fasted, fed, and NAPE-treated animals ($p < 0.001$) ($n = 4–5$ per group).

(H) Quantification of NAPE-induced reductions in the number of cFOS-NPY positive neurons/0.1mm$^3$ in the ARC relative to fasting ($p < 0.0001$) ($n = 4–5$ per group).

(I) Quantification of NAPE-induced reductions in the number of cFOS positive cells/0.1mm$^3$ in the ARC relative to fasting ($p < 0.0001$) ($n = 4–5$ per group).

(J) Representative section at 20X stained for cFOS in a vehicle-treated animal shows low cFOS expression in the PVN.

(K) NAPE treatment significantly increases cFOS staining in the PVN.

(L) Quantification of percent cFOS induction in the PVN by 1000 mg/kg C16:0 NAPE in all animals ($p < 0.008$) ($n = 4–5$ per group).

All data are expressed as mean ± SEM.

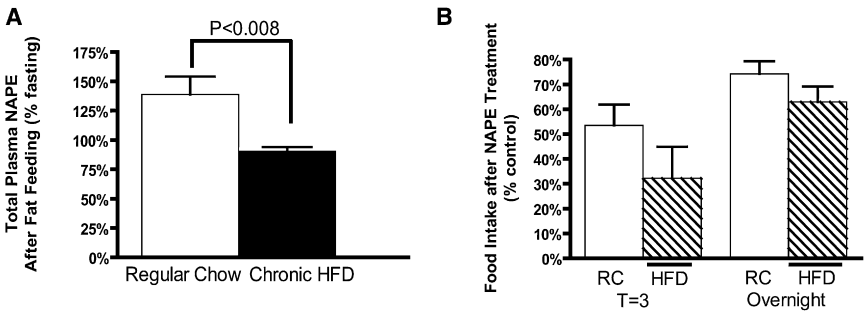


**Figure 6. Chronic High-Fat Feeding Eliminates Postprandial NAPE Secretion without Altering Sensitivity to Its Anorexic Effects**

(A) High-fat feeding significantly increases plasma NAPE at 4 hr relative to fasting in rats maintained on regular chow (open bar), but this effect is absent in animals fed high-fat chow for 35 days prior to the experiment (filled bar) ($p < 0.008$) ($n = 6–14$ per group).

(B) Treatment with 100 mg/kg C16:0 NAPE produces similar reductions in food intake in animals maintained on regular chow and animals fed for 35 days with high-fat chow ($n = 6–8$ per group).

All data are expressed as mean ± SEM.

An unexpected result of this study was that *ob/ob* mice were more sensitive than wild-type animals to the anorectic effects of C16:0 NAPE. Although no definitive explanation for this effect yet exists, one might speculate that leptin may be a permissive signal for NAPE generation or that, similar to our chronically high-fat fed rats, NAPE secretion in response to dietary lipid may be blunted in these mice, thus augmenting their sensitivity to exogenous C16:0 NAPE administration.

Because 20:4 NAPE is the precursor of the NAE anandamide (AEA), a CB1 receptor agonist, we tested C16:0 NAPE in the *CB1*$^{-/-}$ mouse and found that its overnight food intake was reduced to a similar extent to that of wild-type mice treated with an equivalent dose of NAPE, indicating that the anorectic effects of NAPE are independent of the CB1 receptor. We cannot, however, exclude the possibility that CB-type signaling mediates some of NAPE's effects in light of the recent report that GPR55, an orphan G protein-coupled receptor, is potently activated by both PEA, the hydrolysis product of C16:0 NAPE, and $\Delta^9$-tetrahydrocannabinol (Ryberg et al., 2007). This potential overlap with cannabinoid signaling raises interesting questions about the possible involvement of NAPE, or NAPE metabolites, in mediating the rewarding properties of dietary lipid. However, neither direct infusion of PEA into the lateral ventricle nor the pharmacologic elevation of all NAEs in the CNS by inhibition of fatty acid amide hydrolase (FAAH), their catabolic enzyme, affected food intake.

Several of our observations suggest that NAPE may reduce food intake by direct interaction with uncharacterized targets in the central nervous system. First, we found that C16:0 NAPE infusion into the lateral ventricle of cannulated mice, at far lower doses than used in the periphery (75 μg i.c.v. versus 100 to 1000 mg/kg i.p.), significantly reduced food intake relative to i.c.v. injections of an isovolumetric bolus of Liposyn II or an isomolar quantity of phosphatidylethanolamine. Second, elimination of nervous afferents to the brain from the gastrointestinal tract by subdiaphragmatic vagotomy did not block the anorectic effects of NAPE. And, finally, intravenous $^{14}$C-labeled C16:0 NAPE entered the brain and preferentially accumulated in the hypothalamus.

In the ARC, we found that NAPE treatment reduced fasting-dependent induction of the immediate-early gene *cFOS*, which is commonly used as an indirect marker of neuronal activity. A simple interpretation of these data is that NAPE treatment blocks the stimulatory effect that fasting has on cFOS protein production in the hypothalamus and that the observed decrease in these experiments is the product of normal degradation.

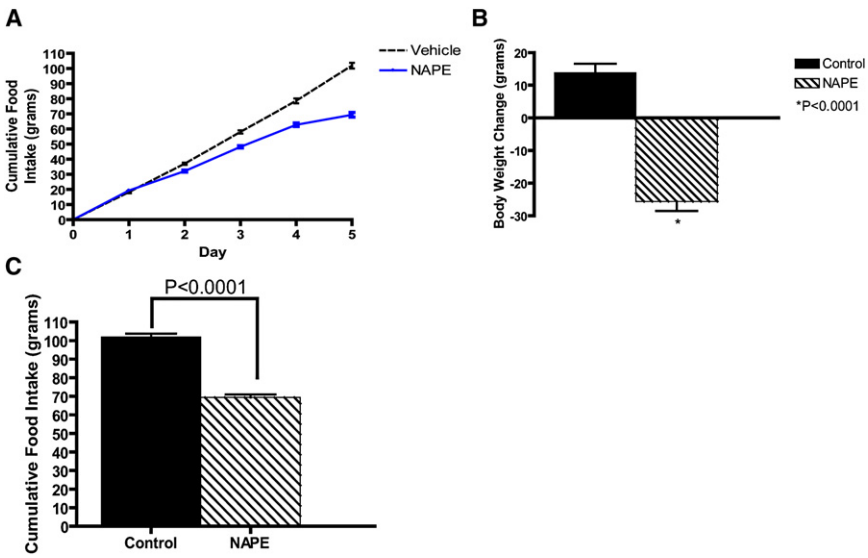


**Figure 7. Continuous NAPE Infusion Reduces Food Intake and Body Weight**

Chronic intravenous infusion of C16:0 NAPE (100 mg/kg-day or 0.07 mg/kg-min) significantly reduces cumulative food intake and body weight in ad libitum-fed rats.

(A) Time course of cumulative food intake in rats treated either with vehicle solution or 0.07 mg/kg-min over 5 days ($p < 0.0001$) ($n = 7–15$ per group).

(B) Comparison of weight change of vehicle treated controls and NAPE treated animals after 5 days of infusion ($p < 0.0001$) ($n = 7–15$ per group).

(C) Cumulative food intake after 5 days in vehicle-treated controls and NAPE-treated animals after 5 days of infusion ($p < 0.0001$) ($n = 7–15$ per group).

All data are expressed as mean ± SEM.

However, these experiments do not provide information regarding the precise transcriptional or electrical changes brought about by NAPE, only that it antagonizes fasting-regulated induction of cFOS in *NPY* neurons and others in the ARC, producing a distribution of cFOS protein much more similar to what is observed in the fed brain. If cFOS expression in this case correlates well with neuronal activity, then the reduction in electrical firing of these cell populations may contribute to NAPE-mediated hypophagia. Future electrophysiology experiments in mice that express GFP in different neuronal subpopulations will help to better characterize the cell types and brain regions responsible for NAPE-elicited changes in behavior.

In opposition to its effects in the ARC, C16:0 NAPE treatment stimulates cFOS protein expression in the hypothalamic PVN and SO nuclei. The anorexigenic peptides CCK-8 (Kobelt et al., 2005), leptin (Emond et al., 2001), and GLP-1 (Turton et al., 1996; Rowland et al., 1997) also stimulate cFOS expression in the PVN, and lesions to this nucleus produce marked hyperphagia, indicating that it may be an important appetite-regulatory region. The induction of cFOS in the PVN and SO by C16:0 NAPE is of further interest because single copy mutations of the transcription factor single-minded 1 (*SIM1*) in mice cause significant cell loss in these regions, obesity, and an impaired ability to sense changes in dietary fat content (Holder et al., 2000; Holder et al., 2004).

A curious property of C16:0 NAPE is its ability to reduce locomotor activity in rodents when given systemically or centrally. Because NAPE treatment did not cause taste aversion or deficits in rotarod performance, these data suggest that it may play a physiologic role in mediating the lassitude typically observed in rodents during the postabsorptive state. There is precedent for interactions between circulating regulators of appetite and the motor circuits in the brain; acute knockdown of the leptin receptor in the ventral tegmental area (VTA) has been shown to increase locomotor activity in rodents (Hommel et al., 2006).

A particularly noteworthy finding of this study was that exposure to high-fat diet for 35 days eliminated the normal postprandial increases in circulating NAPE observed in response to fat feeding. These data suggest that derangements in NAPE secretion associated with chronic high-fat feeding may contribute to the pathogenesis of diet-induced obesity precipitated by overexposure to triglyceride-rich foods. Also, in contrast to leptin, whose anorexigenic properties are rapidly diminished by obesity, C16:0 NAPE was able to still suppress food intake in animals chronically fed a high-fat diet for 35 days.

To characterize the effects of prolonged NAPE treatment on overall energy balance, we chronically infused (i.v.) rats with a low dose of C16:0 NAPE for 5 days, and we observed significant reductions in food intake and body weight relative to vehicle treated rats without adverse effects. These results suggest that chronic C16:0 NAPE treatment is capable of generating a state of negative energy balance over multiple days and merits longer-term studies in rodents and nonhuman primates to examine its potential for treatment and prevention of diet-induced obesity. In conclusion, these data support the hypothesis that circulating NAPEs, synthesized in the small intestine from ingested fat, may be part of an important physiologic negative feedback loop that serves to reduce food intake and arousal after a fat-containing meal.

## EXPERIMENTAL PROCEDURES

### Animals

For all studies in mice, animals of the C57BL6 strain (20–25 g, Charles River, Boston, MA) were maintained on a standard light-dark cycle (12 hr of light followed by 12 hr of darkness) in the Yale Animal Resources Center (YARC) and unless otherwise indicated had ad libitum access to food pellets (Harlan 2018S, Indianapolis IN) and water. Similarly, Sprague Dawley rats were purchased from Charles River and maintained on a 12-12 light-dark cycle in the YARC with free food (Harlan 2018S, Indianapolis IN) access unless otherwise indicated. All animal experiments received prior approval from the Yale University and the University of Cincinnati Institutional Animal Care and Use Committees.

### LC/MS/MS Analyses

All LC/MS/MS method development and analyses were performed on a benchtop Applied Biosystems Instrumentation (ABI) 4000 QTRAP LC/MS/MS system (Foster City, CA), equipped with a Dionex Ultimate capillary liquid chromatography (Sunnyvale, CA) and a DionexFamos autosampler (Sunnyvale, CA) together with a PEAK scientific gas generator (Bedford, MA). Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources were tested with positive- and negative-ion detection. Positive-ion APCI source was found as the most sensitive for both qualitative characterization and quantitative measurements of the NAE, NAPE, and PE metabolites; thus, it was chosen for all method development and applications herein. The autosample temperature was set at 4°C–8°C to minimize the possible degradation of the extracted lipid samples, and aliquots of 5 μl sample solution were injected for each analysis. The tuning and calibration of the mass spectroscopy was performed at unit mass resolution in both positive- and negative-ion modes with a polypropylene glycol (PPG) standard solution. The mass spectra were interpreted and quantified with Analyst software (version 1.4.1).

### High-Fat Diet Screening Study

Eight rats equipped with jugular vein catheters were fasted 24 hr overnight and blood (300 μl) was collected in the fasted state and at 1.5 and 4 hr after refeeding with high-fat chow (59% of calories from fat, AIN-93G, Dyets, Bethlehem, PA). The extracted plasma was analyzed for lipid metabolites, including NAPE and NAE, whose extraction is described in the Supplemental Data.

### Metabolic Cage Experiments

For food intake studies (i.p. treatment), mice were placed in individual cages and allowed to acclimate to the calorimetric apparatus. This comprehensive animal metabolic monitoring system (CLAMS; Columbus Instruments) was used to evaluate activity, food consumption, and energy expenditure.

Immediately prior to lights out at 7:30 PM, ad libitum-fed animals (four in each group/trial) were injected i.p. with lipid control (Liposyn II + 10% Tween 80 or in some cases sterile saline + 10% Tween 80) or the indicated dose of NAPE, at a concentration of 100 mg/kg, 200 mg/kg, 500 mg/kg, or 1000 mg/kg dissolved in the vehicle solution. Food intake was monitored by the CLAMS system for at least the next 12 hr before the experiment was terminated. Solutions were prepared by adding Tween 80 to NAPE, stirring, adding the balance solution and sonicating for 30–60 s.

### Taste Aversion Studies

In brief, three groups of rats (n = 6 per group) were accustomed to a paradigm of serial water deprivation (24 hr) and re-presentation (30 min) until water intake over the presentation period was stable between test days. Once water intake had stabilized, animals were allowed 30 min of access after 24 hr of water deprivation to solutions containing 0.1% saccharin (Sigma, St. Louis, MO). Immediately after removal of the novel flavor solution, animals were injected with vehicle solution, 100 mg/kg or 75 mg/kg LiCl in vehicle solution, or 100 mg/kg

NAPE or 250 mg/kg NAPE in vehicle solution. After a period of recovery days during which water intake stabilized in the deprivation-30 min presentation model, animals were again presented with 0.1% saccharin solution after 24 hr of water deprivation, and their saccharin intake was recorded to test for conditioned taste aversion. The experiment was adapted from Riley and Freeman (2004). For the ICV taste aversion experiment, rats with lateral ventricle cannulas (Taconic Farms, Germantown, NY) were infused with 2.5 μl of NAPE solution (30 mg/ml) dissolved in vehicle solution.

### Intracerebroventricular Infusion Food Intake Studies

Male mice (n = 11–12) were anesthetized with ketamine and xylazine anesthesia and equipped with 33 gauge cannulas (Plastics One, Roanoke, VA, USA). After an incision was carefully made in the scalp and the bregma was located, a small hole was drilled with a 25 gauge needle 1 mm lateral and 0.5 mm caudal to the bregma. The cannula was then secured with dental cement (Lang Dental Mfg. Co., Inc. Wheeling, IL).

Upon recovery, ad libitum-fed cannulated mice were injected with the indicated dose of NAPE in vehicle or vehicle alone (Liposyn II + 10% Tween 80 in 2.5 μl) immediately before lights out, and food intake was recorded with the complete metabolic monitoring system described above. An identical protocol was used for i.c.v. treatment with dioloeylphosphatidylethanolamine (DOPE) and PEA.

### URB597 Food Intake Studies

In these studies, 24 hr-fasted mice were injected with either vehicle (saline + 10% Tween 80) or 0.3 mg/kg URB597 in vehicle, and food intake was recorded overnight.

### *NPY*-GFP and cFos Immunohistochemistry

For the fasting-induced cFOS and cFOS-NPY colocalization experiment, overnight fasted mice were treated 1 hr prior to sacrifice with 1000 mg/kg NAPE (in Liposyn II + 10% Tween 80) or lipid control (Liposyn II + 10% Tween 80). Upon sacrifice mice, were subjected to intracardiac perfusion with a solution containing 4% paraformaldehyde, 15% picric acid, and 0.1% glutaradlehyde, and the dissected brains were postfixed overnight in an identical solution lacking glutaraldehyde. After several rinse steps, the brains were cut in 50 μM sections with a vibratome and washed in 0.1 M phosphate buffer (PB). After treatment in 1% $H_2O_2$ for 12.5 min, the slices were washed for 30 min in PB prior to incubation for an additional 12.5 min in a solution containing 20 μl/ml Triton X-100/ml. After this step, slices were washed with PB for 30 min. Next, slices were incubated overnight with a rabbit antibody generated to cFOS (Oncogene, San Diego, CA) at a 1:6000 dilution. After another wash for 1 hr in PB, the secondary antibody was added (RbAlexa Flour 594, 1:200) (Molecular Probes, Carlsbad, CA).

### Lipid and Dextrin Infusion Experiments with Lymph Sampling

SD rats fasted overnight (1700–900 hrs) were cannulated with tubes in the duodenum and lymphatic duct. After surgery, 10% glucose saline was infused in the duodenum at 1.5 ml/hr until 1700 hrs on the surgery day. So that tubes could be kept clear, 0.9% saline was given at 1.5 ml/hr until morning. In the morning, fasting lymph was collected before the treatment. Two different infusions were given to different groups of animals after fasting lymph was collected: (1) lipid treatment, in which 2.2 ml 20% intralipid (Liposyn II, Hospra Inc., Lake Forest, IL) plus 0.8 ml 0.9% saline was infused or (2) dextrin treatment, in which 3.69 g in 10 ml PBS was infused. A bolus of either intralipid or dextrin (3 ml) was given for each rat while 0.9% saline infusion was stopped for 30 min. After 30 min, continuous infusion of 0.9% saline was given at 3 ml/hr for the rest of the 6 hr infusion, and lymph samples were collected from the lymphatic duct at 0.5, 1, 2, 3, 4, 5, and 6 hr.

### Protein Infusion Experiments with Lymph Sampling

SD rats were fasted overnight (1700–900 hrs) and were cannulated with tubes into the duodenum and lymphatic duct the next morning. After surgery, 5% glucose saline was infused into the rat duodenum at 3 ml/hr until 1700 hrs that day, after which time 0.9% saline was infused at 3 ml/hr until the next morning. At that time, fasting lymph was collected for 1 hr during the continuing saline infusion. Next, a bolus of 3 ml infusion (0.8 g albumin in 3 ml) was injected into the rat duodenum as the normal saline infusion was discontinued for 30 min. After 30 min, continuous infusion of 0.9% saline was resumed at 3 ml/hr. Lymph samples were collected at the intervals indicated (Figure 1) and centrifuged at 3000 rpm for 10 min to remove the contained cells.

### NAPE Concentration in Stomach and GI Tract

SD rats (n = 8) were fasted overnight and half were refed for 90 min with high-fat chow (59% of calories from fat, AIN-93G, Dyets, Bethlehem, PA). At this time, all animals were sacrificed, and their GI tracts were sectioned and flash frozen in liquid nitrogen. Tissues were then ground while frozen and NAPE was extracted.

### NAPE Secretion Experiment in DIO Rats

Two groups of rats were maintained on either regular chow or high-fat diet (55% kcal from fat, Harlan Teklad, Madison, WI) for 35 days, at which time they were fasted overnight and refed with a different high-fat chow (59% of calories from fat, AIN-93G, Dyets, Bethlehem, PA) to control for taste neophobia effects, and plasma NAPE levels were measured throughout the re-feed via jugular vein catheters.

### *ob/ob* Feeding Experiments

Ad libitum-fed *ob/ob* mice (Jackson Labs, Bar Harbor, ME) were treated with i.p injections of 500 mg/kg C16:0 or vehicle, and overnight food intake was monitored.

### *CB1*$^{-/-}$ Feeding Experiments

*CB1*$^{-/-}$ mice were fasted for 24 hr and refed with regular chow after treatment with either 500 mg/kg C16:0 NAPE or vehicle.

### Radiolabeled NAPE Infusions

Rats were infused i.v. with 2.5 μCi of N-palmitoyl-1-$^{14}$C phosphatidylethanolamine (American Radiolabeled Chemicals, St. Louis, MO). After 4 hr, brains and hypothalami were removed, cleaned, and weighed, and radioactivity was quantified with a scintillation counter.

### Chronic NAPE Infusion Studies

Rats equipped with jugular and femoral vein catheters were placed in free-ranging chronic infusion harnesses and coupled to infusion pumps, receiving 0.07 mg/kg-min C16:0 NAPE or vehicle (5% Tween 80, 5% propylene glycol, 90% sterile saline) continuously in the jugular vein for 5 days, while body weight and food intake were monitored.

### Lipid and Dextrin Infusion Experiments with Plasma Sampling

SD rats fasted overnight (1700–0900 hrs) were cannulated with tubes in the jugular vein and duodenum. After surgery, 10% glucose saline was infused in the duodenum at 1.5 ml/hr until 1700 hrs on the surgery day. So that tubes could be kept clear, 0.9% saline was given at 1.5 ml/hr until next morning. Next morning, fasting blood (250 μl + 3 μl heparin) was collected before the treatment. Two treatments were given to the animals after fasting blood was collected: (1) lipid treatment, in which 2.2 ml 20% intralipid (Liposyn II, Hospra Inc, Lake Forest IL) plus 0.8 ml 0.9% saline was infused, or (2) dextrin treatment, in which 3.69 g in 10 ml PBS was infused. A bolus of either intralipid or dextrin (3 ml) was given for each rat while 0.9% saline infusion was stopped for 30 min. After 30 min, continuous infusion of 0.9% saline was given at 1.5 ml/hr for the rest of the 6 hr infusion, and plasma samples were collected at 0.5, 1, 2, 3, 4, 5, and 6 hr. A total volume of 200 μl plus 3 μl Heparin was collected from each rat per sample. At the end of the 6 hr collection, final plasma (3 ml) was collected from the jugular vein.

### Rotarod

A group of ten mice were acclimated to the experimental device for 30 min at 2 rpm. Upon completion, all animals were again placed on the rotarod, which was then accelerated by 0.01 rpm/s until all had fallen from the rod. On the test day 3 days later, half of the mice were injected with 1000 mg/kg C16:0 NAPE and the other half with a lipid control 1 hr prior to the experiment. The rotarod test was then repeated with the same accelerating program, and the time at

which each animal fell from the rod was recorded. This test was repeated five times.

### Statistics

All data are expressed as mean ± SEM. Data were analyzed with unpaired t tests, paired t tests, a Wilcoxon signed rank test, repeated-measures two-way ANOVA with Bonferroni post-testing, and a one-way ANOVA followed by a Neuman-Keuls multiple comparison test when appropriate with GraphPad Prism (San Diego, CA).

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01380-9.

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